File Number: 25943

Applicant Information

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Project Information

File Number: 25943
Application Status: Application Complete
Project Title: Using earplugs and baleen to assess lifetime stress profiles in whales
Project Status: New
Previous Federal or State Permit/Authorization: 20532

Permit/Authorization Requested:	• MMPA/ESA Parts permit - Issued
Where will activities occur?	US Locations including offshore waters
Research Timeframe:	**Start:** 11/23/2021 **End:** 11/30/2026
Sampling Season/Project Duration:	This project requires using archived whale parts housed in museums and/or universities as well as recently collected samples from stranded whales within the U.S. and in foreign countries. This is ongoing research that was developed in 2011 and is expected to continue indefinitely.
Abstract:	The purpose of this research is to receive, import, and export parts, including earplugs, baleen, and teeth, of baleen whales (Mysticeti; Balaenopteridae, Balaenidae, Eschrichtiidae) and sperm whales (Physeteridae), from museum holdings as well as from stranded whales, subsistence hunted, and fisheries bycatch from the U.S and abroad. We have determined that these samples represent a marine mammal matrix capable of recording and archiving anthropogenic and physiological data. Like all mammals, baleen whales excrete wax into their ear canals, where it accumulates over their entire life (~20 to 100+ yrs) forming an earplug. Baleen is also a keratin matrix and represents a relatively short-term high-resolution matrix to validate chemicals recovered from earplugs. Recent published data measured in blue whale earwax plugs suggests that lipophilic compounds such as stress hormones and pesticides accumulate in this waxy ester and lipids rich matrix. Earplugs contain light and dark layers, which are thought to be associated with annual to biannual activity and have been used to estimate an individual's age, similarly to tree ring-dating techniques. Therefore, this method demonstrates that long-lived whales are active marine monitoring systems with the ability record and archive data via earplug. The focus species will include obtaining parts from Blue, Gray, Fin, Minke, humpback, sperm, Bryde's, Rice's, N. Atlantic right, N. Pacific right, S. right, Bowhead, and sei whales, and unidentified cetacean species. Bowhead whale parts may also be acquired during subsistence hunts in Barrow Alaska. Marine mammal parts may also be acquired from preserved museum holdings or from necropsies of stranded whales in foreign countries, fisheries bycatch, or legal subsistence hunts. We expect a maximum of parts from 100 individuals from each species listed above per year. Samples will also be imported from museums and universities worldwide. This permit duration is requested for 5 years.

Project Description

Purpose: Marine mammals, especially baleen whales, are known to be ocean engineers and sentinels (O'Hara and O'Shea 2005; Hoekstra et al. 2003; Reijnders et al. 1999; Trumble et al. 2018; Moore 2008, Grebmeier et al. 2006). As such, over the past quarter century, the goal is to improve our understanding of the biological effects of manmade or environmental stressors on marine mammals, especially large whales. However, despite continued efforts into identifying, and in some cases mitigating, the cause and effect of stressors have increased over the past 40 years. In 2017, the National Academy of Sciences (NAS) released a report describing the current state and future approaches of understanding the cumulative effects of multiple stressors (including natural and anthropogenic sound) on marine mammals. In this report, earplugs and baleen were highlighted as a novel tool for answering this complex multi-faceted challenge due to their ability to archive several lipophilic compounds longitudinally. Further, limitations of current approaches for assessing the impact of two or more stressors on a population or ecosystem, specifically were addressed, such that the assumptions that stressor effects are additive (e.g. synergistic interactions) as well as existing data sets based on opportunistic or single-point sampling. The limitations of these approaches often limit the scope and questions that can be addressed and often exclude the assessment of multiple stressors. Therefore, datasets focusing on single-point sampling, while contributing to the current knowledge, have also stalled progress, such that cumulative risk from exposure to multiple stressors cannot be adequately predicted for individual marine mammals or their populations.

Given the difficulties with assigning the impacts of multiple stressors that vary as a function of space and time, especially as they relate to a migratory whale, recent mean baseline-corrected cortisol data identified large-scale anthropogenic activities as physiological stressors in baleen whales (specifically fin, blue and humpback whales) in the Northern Hemisphere during the 20th and early 21st centuries (Trumble et al. 2018). In this study, several variables (and their interactions) were modeled against stress (species, sex, age, yearly whale harvest, and sea surface temperature anomalies) to provide the first study to assess the effect of several stressors on the overall stress of large whales. A critical component to the assessment of the effect of exposure to multiple stressors is determining the natural variability inherent within animals and among populations. Using baseline-corrected data from lifetime stress/stressor profiles, Trumble et al. (2018) determined the natural variability of stress profiles, and, when combined, identified potential responses to sub-lethal stressors. These responses could be modeled against known stress datasets to identify possible associations.

Typically used as a measure of aging in cetaceans (order Mysticeti), ear plugs produce alternating yearly (humpback whales, Gabriele et al. 2009; blue whale, Trumble at al. 2013) dark and light lamina and remain undisturbed during the lifetime of the animal. An earplug is a two-part keratin and lipid structure in the auditory meatus of mysticetes whales (Purves 1955) that appears to increase with age. While the literature on earplug ageing has often used vague and inconsistent terminology, Roe (1968) clearly established the link between mysticete migration patterns and lamina formation by examining the most recently formed lamina in fin whale earplugs collected in all months of the year, finding a high fat content, and keratinized epithelial. Thus, earplugs may provide an excellent repository of lipophilic substances (hormones) and chemicals. Our lab has also worked with baleen as a higher-resolution and shorter time-based matrix. We have been successful in obtaining the same chemical constituents from baleen and sperm whale teeth.

Problem Statement. Chemical trends and profiles that have been reconstructed from matrices such as sediment and ice cores have provided a wealth of information regarding contaminant behavior and environmental fate. Physiological or health trends can be derived from short-term indices such as serial blood samples to longer periods such as blubber. Regardless, these indices provide no more than a few months of historic data. This proposed research seeks to validate our existing methods to detect, quantify, and reconstruct anthropogenic and physiological profiles in whales using earwax plugs and validate with baleen from the same animal. Our technique correlates possible age with (decades) chemical exposure and physiological data for a single individual whale.

Research Goals, Assumptions and Objectives
Overall Research Goal. To chronologically profile anthropogenic (bulk stable isotopes; C,N,S,O), contaminants, and physiological stress and reproductive hormones from baleen whale whale earplugs and baleen as well as from sperm whale teethto determine influence of anthropogenic and environmental stressors.

Project Assumptions. The ability for whale earplug/baleen/teeth to chronological record and archive anthropogenic and physiological data is based on the following assumptions.
Lipophillic biological and anthropogenic analytes (hormones, stable isotopes, contaminants) accumulated will accumulate in waxy ester and lipid/keratin rich matrix of baleen whale earplugs and baleen (Trumble et al. 2013; Trumble et al. 2018; Mansouri et al. 2021; Winfield et al. 2020; Crain et al. 2020).
Lipophillic biological compounds, such as stress or reproductive hormones will accumulate and quantified in the teeth of sperm whales.
Lipophillic compounds and stable isotopes measured in earplug will be achieved within the laminated earwax layers, baleen, and sperm whale teeth (Trumble et al. 2013; Trumble et al. 2018, Winfield et al. 2020, Mansouri et al. 2021).

Objectives.
1. Determine age-related stress (cortisol, corticosterone) and reproductive hormone (progesterone, testosterone, estradiol, estrogen) profiles (6-12 month resolution) from archived and stranded mysticete/odontocete samples (earplugs, baleen, teeth) from the US and abroad (international).
2. Reconstruct longitudinal contaminant profiles/exposures from archived and stranded mysticete/odontocete samples from the US and abroad.
3. Quantify additional metabolic markers from samples collected from stranded and museum specimens in the US and abroad.

4. Compare stress levels of extant organisms exposed to specific contaminants and/or environmental changes to baseline levels from samples collected from stranded and museum specimens in the US and abroad.
5. Examine possible correlations among physiological responses, contaminant exposure and environmental changes (stable isotopes) from samples collected from stranded and museum specimens in the US and abroad.

Rationale and Significance
This permit request builds on the success of our previously funded projects, which identified individual baseline and reconstructed lifetime profiles of stress-related hormone levels in baleen whales using earplugs and baleen. The NAS reported the significance of this line of research as “This is the only shortcut found by the Committee for retrospective studies of health and vital rates where one can use tissue from dead animals to study these relationships from birth to death.” The NAS report also specified, “If large enough samples of earplugs can be recovered and analyzed for health and vital rates, this could enable a new way to evaluate the relationship between these critical parameters”. Our team developed and has been at the forefront of this research while advancing on numerous fronts; 1) the continued analysis of earplugs from various baleen whale species for stress, 2) expanding the number of stressors examined, 3) the development of collaborations with national and international museums including opportunities for public outreach, and 4) development of collaborations with stranding networks around the world. During our initial research, the focus included hormones (stress and reproductive) and contaminants; however, our research team has expanded current analytical efforts for whale earplugs to include stable isotopes (13C and 15N), inorganic contaminants (i.e. metal including Hg and methylmercury) and chiral analysis (i.e stereoisomers) and DNA extraction (stock assignment). Data collected as part of the previous project highlight significant variation in stress both spatially and temporally (~past 150 years) therefore with the addition of more samples and analytical techniques, these stressors can be isolated. This top-down approach examines the aggregate exposure from biogenic, anthropogenic, and oceanic/ecosystem stressors to help provide a more comprehensive evaluation of health.

The research of reconstructing analyte profiles using whale parts (earplugs, baleen, teeth) is bona fide because unlike point-sampling (blood, hair, feces) the years to lifetime profiles provide a unique assessment of physiological response to known environmental or anthropogenic activities. From our previous work and permit, we have published ten+ peer-reviewed manuscripts (with a few more in the pipeline) and therefore feel confident that future work will be published in a refereed scientific journal. While this research contributes to the knowledge of large cetaceans from a purely scientific basis, this research has also validated the collections and sampling for the museums which have housed these samples, in some cases for several decades. I have been personally contacted by curators, thanking us for giving credence to their mission. This research line has been successfully funded by federal agencies as we are resolving interactions between large marine mammals and humans.

Reconstructing lifetime stress profiles (i.e. birth to death) using baleen whale earplugs, research developed by the PI, is uniquely capable of assessing stress trends temporally and spatially. This reconstruction technique combines age estimates (i.e. counting light and dark lamina) with chemical or elemental analysis (i.e. hormones) of each waxy layer (lamina). Moreover, our team has been able to acquire additional tissues such as baleen and teeth (baleen hormone reconstruction was developed in our labs several years ago) to analyze against the corresponding earplug as a means of providing additional scientific rigor and validation. Therefore, the long-term goal is to examine lifetime cause-effect relationships for these sentinel species, specifically quantifying intrinsic and extrinsic responses to natural and anthropogenic stimuli over time and therefore providing an opportunity to assess changes through time in both the environment as well as its impact on this species.

Baleen whales are the only extant species to carry earplugs and baleen, the two tissues used to reconstruct up to lifetime analyte profiles. This coupled with a lack of knowledge for large whale cause/effect associations is the rationale for using samples collected from ESA-listed species. Our group has published several papers involving lifetime hormones and stable isotope profiles, providing contaminant exposure differences between ocean basins, reproductive success with age, and stress patterns as it relates to anthropogenic pressures. These results have already been applied to general population models as it pertains to the conservation efforts to current baleen whale populations.

Justification for sample size requested.
While unlikely, we may gain access to 100 samples per year (100 individuals), from museums and/or strandings. Because this research has generated enthusiasm, we have been granted access to several earplug archived holdings. For example, we work closely with the Smithsonian Museum of Natural History; they currently have hundreds (maybe over a thousand) earplugs, half of which are not identified to an individual whale (only species and date). We may be offered the unidentified plugs for hormone and genetic analysis which would amount to needing a permit to accommodate this quantity of samples. If available, we will collect the corresponding baleen to each animal earplug. Baleen plates are made of keratin and our lab has been able to recover hormones and stable isotopic signatures from each layer. We use this to age validate etc what is recovered from the earplug.

Previous findings
Below is a list of currently accepted or published data resulting from this research:

*Mansouri, F., Winfield, Z.C., Crain, D.D., Morris, B., Charapata, P., Sabin, R., Potter, C.W., Fulton, J., Trumble, S.J., S. Usenko. 2021. Evidence of multi-decadal behavior and ecosystem-level changes revealed by reconstructed lifetime stable isotope profiles of baleen whale earplugs. Science of the Total Environment 757 (2021) 143985 https://doi.org/10.1016/j.scitotenv.2020.143985
*Crain, D. D. Thomas, A., Mansouri, F., Potter, C.W., Usenko, S. and S.J. Trumble. Hormone comparison between right and left baleen whale earplugs. 2020. Conservation Physiology, Volume 8, Issue 1, https://doi.org/10.1093/conphys/coaa055
*Mansouri, F., Crain, D.D., Winfield, Z.C., Sabin, R., Potter, C.W., Zhang, R., Trumble, S.J., S. Usenko. 2020. A lipid normalization model for the analysis of stable isotopes in baleen whale earplugs. Marine Mammal Science DOI: 10.1111/mms.12756.
*Crain, D.D., Usenko, S., Mansouri, F., Winfield, Z.C., Zerbini, A.N., Gabriele C., Hering, A.S., C., Sabin, R., Potter, C., Trumble, S.J. 2021. Measuring progesterone in baleen whale earplugs: reproductive parameters, patterns of senescence, and modeling rate of increase Communications Biology (Nature). Accepted May 2021.
*Winfield, Z.C., Crain, D.D., Mansouri, F., Potter, C.W., Sabin, R., Trumble, S.J. and S. Usenko. 2020. Eighty Years of Chemical Exposure Profiles of Persistent Organic Pollutants Reconstructed Through Baleen Whale Earplugs. Science for the Total Environment. https://doi.org/10.1016/j.scitotenv.2020.139564
Trumble, S.J., Norman, S.A., Crain, D., Mansouri, F., Winfield, Z., Sabin, R., Potter, C.W., Gabriele, C., and S. Usenko. 2018. Baleen whale cortisol levels reveal a physiological response to 20th century whaling. Nature Communications DOI:10.1038/s41467-018-07044-w.
Lysiak, N.S., Trumble, S.J., Knowlton, A.R. and M. Moore. 2018. Characterizing the Duration and Severity of Fishing Gear Entanglement on a North Atlantic Right Whale (Eubalaena glacialis) Using Stable Isotopes, Steroid and Thyroid Hormones in Baleen. Frontiers in Marine Science 168(5) 1-13. https://doi.org/10.3389/fmars.2018.00168
Usenko, S., Trumble, S.J. and N. Lysiak. 2015. GC–MS and UHPLC–MS-MS Analysis of Organic Contaminants and Hormones in Whale Earwax Using Selective Pressurized Liquid Extraction. Chromatography (2): 40-44.
*Robinson, E.M., Jia, M., Trumble, S.J., and S. Usenko. 2015. Selective pressurized liquid extraction technique for halogenated organic pollutants in marine mammal blubber: A lipid-rich matrix. Journal of Chromatography A 1385, 111-115.
*Robinson, E. M., Trumble, S.J., Subedi, B., Sanders, R. and S. Usenko. 2013. Selective pressurized liquid extraction of pesticides, polychlorinated biphenyls and polybrominated diphenyl ethers in a whale earplug (earwax): A novel method for analyzing organic contaminants in lipid-rich matrices. Journal of Chromatography A. 1319:14-20.
Trumble S.J., Berman-Kowalewski, M., Potter, C. and S. Usenko. 2013. Blue whale earplug reveals lifetime contaminant exposure and hormone profiles. Proceedings of the National Academy of Sciences. 110 (42)16922-16926. DOI:10.1073/pnas.1311418110

Because of the permit issued and samples collected, several publications and samples have been produced and archived. Below is a synopsis of findings.

Reproductive hormones in whale earplugs
Stress and reproductive hormones were extracted from individual lamina from each earplug. Baleen whale earplugs were bisected longitudinally, and the lamina counted for aging, after which each lamina was manually separated from the other. These laminae were homogenized and stored under nitrogen at -80°C until hormone extraction. Hormones were extracted where 15-25 mg of homogenized earplug lamina was vortexed and centrifuged in diethyl ether, pipetting off the supernatant and blowing down to dryness. The extract was stored under nitrogen at -80°C until hormone analysis. Hormone analysis was carried out via enzyme-linked immunosorbent assays (ELISA, ENZO Life Sciences, progesterone ADI-901-011) to assess concentrations in pg/g sample for progesterone and read on a DTX 880 (Molecular Devices). Hormone concentrations were corrected by sample mass (weighed to nearest 0.01 mg), which ranged from 15-25 mg.

Estimating reproductive events using whale earplugs: Validation and sensitivity analysis
Pregnancy events, rates of increase (ROI), and age-specific fecundity were identified from progesterone concentrations in lamina. Error was calculated using standard error, SE, where s is standard deviation and n is number of earplugs or individuals. Reproductive senescence was determined from individual trends in age and progesterone Z-scores. Pregnancies were identified by first calculating percent change progesterone, herein delta progesterone, from the preceding point from the raw progesterone concentration data. This reduced inter-lamina variability within a single earplug and highlighted large increases of absolute progesterone concentrations. Progesterone values were normalized by calculating lamina Z-scores for each individual earplug, where the distance in standard deviations from the mean is calculated for each point:

([delta progesterone]-[x ?])/s Eq. 1

where [delta progesterone] is the delta progesterone in percent for that lamina, x ? is the mean delta progesterone for that earplug, and s is standard deviation of delta progesterone for that earplug, respectively. This normalization allowed the authors to assess how progesterone changed across time while controlling for variability in progesterone excretion, where the average hormone concentration for each whale corresponds to a Z-score of zero.

To estimate plausible pregnancies from the laminae, a spectral analysis was completed. The goal of a spectral analysis is to determine if there are any identifiable cycles in the time series. Applied to the standardized delta progesterone values of a given whale, a cycle would correspond to the pregnancy interval, which is the length of time between sharp increases in delta progesterone. Given that a whale's lifetime may be short, there can be substantial uncertainty in identifying these cycles, and there can be strong inter- and intra-species variability as well.

The period identified by the spectral analysis for each whale was used to identify the largest number of plausible peaks in a whale's delta progesterone Z-score, indicating a possible pregnancy during this period. For a given whale, its total sample size was divided by its smallest period. For example, the minke whale has 90 six-month values. It has important periods at 3 and 4.3 years. Thus, 90 divided by 3 is 30, indicating at most 30 pregnancies during its lifetime (“maximum pregnancies”). Next, delta progesterone Z-scores were ranked from smallest to largest and the largest 30 values were taken as possible pregnant years. Possible pregnant years were removed if they occurred in consecutive six-month periods. Namely, if two large delta progesterone Z-scores occur next to each other, the smaller of the two was eliminated as a potential pregnant year. Delta progesterone Z-scores smaller than zero were also eliminated (“estimated pregnancies”).

Using pregnancies over each individual lifetime identified from the data-driven approach described above, age at first pregnancy was estimated by identifying the first peak occurring within the range of published age at first parturition, subtracting one year for gestation. Pregnancy interval is defined as the smallest period identified in the spectral analysis (“pregnancy interval (1/?)”). Time between estimated pregnancies is the length of time between each estimated pregnancy for each individual. Pregnancy rate was calculated as:
(?pregnancies)/(age-AFP) Eq. 2

where ?pregnancies are the total number of pregnancies of an individual, age is estimated age at death of the individual (derived from the earplug laminae), and AFP is age at first pregnancy. Time between estimated pregnancies, age at first parturition, and pregnancy rate were derived for each whale. These results demonstrate the inherent variability in individual pregnancy rates. Progesterone peaks within a lamina were classified as true pregnancies and not as ovulation/estrous or pseudopregnancies because of the consistent elevation in hormones over the six-month period.

Pregnancies estimated from this spectral analysis method correspond with pregnancies in two humpback whales for which sighting data is available (sightings n = 9)3. Pregnancy events were estimated by taking the year a calf was sighted, subtracting the year the whale had the nearest lamina assigned as a pregnancy, and subtracting 1 for gestation. For instance, a calf of humpback whale (ID# 1020) was sighted in 1986, and the nearest lamina assigned as a pregnancy was excreted in 1985, from which one was subtracted to account for gestation, resulting in zero. This indicates the lamina shows a pregnancy one year before the calf was sighted. Furthermore, calculated time between estimated pregnancies, age at first parturition, and pregnancy rates are similar to published values. As such, pregnancy rates were treated as equivalent to birth rates, which is why these pregnancy rates were used as birth rates in the ROI calculations.

Pilot study: average rates of increase (ROI) and patterns of senescence using whale earplugs
Using the model, M1, for fin whales, a calf survival rate of 0.806 and non-calf survival rate of 0.955 were used. To incorporate data generated from this study into M1 a mean pregnancy rate of 0.48 and mean age at first parturition at 7.7 years were used (the average ROIM1). Patterns of senescence are not included in M1. ROI is expressed as yearly percent population increase. M1 requires tp (age at first parturition), S (non-calf survival rate), Sc (calf survival rate), p (birth rate), qf (sex ratio at birth), and r (intrinsic growth rate):

e^(t_p?r)= e^((t_p-1)?r)?S +pq_f?S_c?S^((t_p-1)) Eq. 3

Potential use of earplug progesterone in estimating ROI for population trajectories

To include patterns of senescence in the calculations of ROI, the Lotka equation, M241 was used:
1 =?_(?=1)^???e^(-r?) l(?)b(?) ? Eq. 4

Where l(?) is age-specific survival at age (?), b(?) is age-specific fecundity at age (?), r is intrinsic growth rate, and ? is age of reproductive senescence. Progesterone Z-score was used because baleen whales reproduce comparatively slowly, making it difficult to assess how pregnancy rate is affected by age at the population level when only using a few individuals. Therefore, the comparison between M2 and M1 is an exercise to exhibit the potential of M2 when baleen whale earplug sample size increases to examine lifetime reproduction in these animals. Essentially, the only difference between the parameters for these two equations is using age-specific fecundity/reproductive senescence (M2) or not (M1).

Statistics & Reproducibility
To assess differences in progesterone concentration and delta progesterone over the whales' lifetimes, a linear mixed-effects model, or LMM, was used in R with package nlme and function lme. Whale ID was the random effect and age by whale ID was incorporated into the LMM to control for any autocorrelation that may appear in the data due to the nature of multiple measurements from the same individual over its lifespan. An ANOVA was used to examine the impact of earplug storage (museum or frozen) on mean percent binding in progesterone assays.
This pilot study focused on the general effect of age on pregnancy rate. However, progesterone Z-score was used instead of pregnancy rate, because long-lived species that

reproduce every 2 to 3 years do not provide enough resolution in pregnancy rate data at low sample sizes of individuals. To assess this effect, a generalized additive mixed-effects model (GAMM) was used where age was smoothed as the fixed effect with three knots defined to mimic the natural biological signature of reproductive senescence. Individual whale ID was assigned as the random effect. From this GAMM structure progesterone Z-score was converted to pregnancy rate using the mean fin whale pregnancy rate (0.59) as the maximum pregnancy rate at age 25. On either side of age 25 pregnancy rate decreased to match the pattern of the GAMM.

Hormone comparison among earplugs, blubber, and baleen from individual baleen whales
Hormone analysis among tissues: baleen whale earplugs, baleen, and blubber were sampled from three juveniles and two adults (N=5): one gray whale (Eschrichtius robustus), two fin whales (Balaenoptera physalus), and two humpback whales (Megaptera novaeangliae, Table 1).
Hormone analysis was carried out via enzyme-linked immunosorbent assays (ELISA, ENZO Life Sciences, cortisol ADI-900-071, progesterone ADI-901-011, testosterone ADI-901-065) to assess concentrations in ng/g for cortisol, progesterone, or testosterone. Color change was read on a DTX 880 (Molecular Devices). To account for different hormone baselines among the biological matrices, all hormone concentrations were Z-score normalized.

Bulk carbon (?13C) and nitrogen (?15N) stable isotopes were measured for each corresponding centimeter of baleen sampled. Stable isotope periodicity was used to determine baleen growth rate for individual whales. Specifically, the length of baleen growth required for a full period using ?13C or ?15N was used to calculate growth rate of baleen over a year of time, allowing researchers to match up earplugs and baleen by representative time. Periodicity was defined as either ?13C or ?15N completing a full cycle, either at a low point or high point (Fig. 1).
The results of the stable isotope ratio are presented as delta (?) values per mil (‰) relative to the standard as defined by the following equation:
?X=(((R_Sample)/R_Standard)-1)*1000
Where X is 13C or 15N, and R corresponds to the 13C/12C or 15N/14N ratio of the samples or standard. The standards were Vienna Pee Dee Belemnite (V-PDB) calcium carbonate for 13C and atmospheric nitrogen for 15N.
In the event periodicity was not evident from ?13C or ?15N patterns, growth rates of baleen were determined as follows: 17 cm/year as the mean of the range of fin whale baleen growth rates as 11-23 cm/year (Aguilar et al., 2014) and gray whale baleen growth rates estimate one baleen plate represents 1-1.5 years growth or in the case of the length of whale ID# 1100, 8 cm/year.

Blubber
Full thickness blubber samples from each individual whale, taken from the skin to immediately distal to the muscle, were separated into three equal portions: distal (adjacent to the epidermis), medial, and proximal (adjacent to the muscle layer). These blubber portions were homogenized and lipid-extracted using a modified Soxhlet method (Soxtec 2043, FOSS). Lipid extract was dried under a stream of nitrogen, weighed (nearest 0.01 mg) and stored at -80 °C before hormone analysis. To assess possible trends of hormone concentrations in distal, medial, and proximal blubber, the hormone concentrations for these stratifications were averaged across individuals and compared. Full thickness blubber sample hormone concentrations were determined for individuals by calculating a mean from the hormone concentrations of the distal, medial, and proximal blubber sections (Table 2).

Blubber Data Analysis
To compare hormone concentration among tissues, Z-score normalization of cortisol, progesterone and testosterone values was used for baleen whale earplugs and baleen. However, because blubber represents a single time point, absolute values (ng/g lipid) were used when comparing among earplug, baleen, and blubber hormone concentrations. Baleen analyzed at centimeter intervals is reported to indicate a resolution of weeks to months, baleen hormone concentrations were averaged over a six-month period based on growth rate from stable isotopes for comparison with earplug lamina (Fig. 1). After calculating the six-month mean hormone concentration, Z-scores were calculated again. A Pearson’s product-moment correlation was performed using the function cor.test for all correlations that were tested (R Core Team, 2018). Significant outliers were removed

with the Grubb's test using the "outliers" package and grubbs.test function.

Results

Hormone assays

Mean coefficient of variation (CV) for all hormone assays were 9.9 ± 5.3% for earplugs, 8.5 ± 7.1% for baleen, and 8.5 ± 6.7% for blubber (mean ± standard deviation).

Baleen plate growth rates

Growth rates of baleen calculated using stable isotopes yielded varying rates among animals sampled (Fig. 1, Table 1). For fin whale ID# 1015, the baleen growth rate was estimated at 18 cm per year (Fig. 1A), The adult humpback whale (ID# 1024) baleen growth rate was estimated at 12-18 cm/year for which we used 12 cm/year due to the clearer periodicity from ?15N (Fig. 1C). The juvenile humpback whale (ID# 1025) baleen growth estimate was 18-22 cm/year; we chose 22 cm/year as the growth rate for this individual's baleen. The juvenile fin whale (ID# 1019) and adult gray whale (ID# 1100) did not show any periodicity in baleen stable isotopes (Fig. 1B, 1D); therefore 17 cm/year and 8 cm/year were used for baleen growth rate, respectively.

Mean hormone concentrations in blubber, baleen, and earplugs

Blubber. There was no difference for mean absolute blubber hormone concentrations by depth in cortisol or reproductive hormone values (ANOVA, $F = 1.1$, $p = 0.36$). In full thickness blubber, the adult female gray whale (ID# 1100) and adult male humpback whale (ID# 1024) had significantly higher cortisol concentrations in full thickness blubber as compared to juvenile whales (946 ± 374.4 and 111.3 ± 81.8, respectively, ANOVA, $F = 9.5$, $p = 0.009$) (Table 2). The adult female gray whale (ID# 1100) had significantly higher progesterone than juveniles (21.6 ± 10.6 ng/g lipid, ANOVA, $F = 24.4$, $p = 0.002$) whereas the adult male humpback (ID# 1024) and juvenile male fin whale (ID# 1015) could not be differentiated based on their blubber testosterone alone (ANOVA, $F = 1.9$, $p = 0.24$) (Table 2).

Baleen. Hormone concentrations for baleen plates revealed the adult female gray whale (ID# 1100) and adult male humpback whale (ID# 1024) had significantly higher cortisol concentrations than juvenile whales (62.2 ± 40.7 and 35.1 ± 12.9 ng/g lipid, respectively, ANOVA, $F = 20.7$, $p = 1.2$ x 10-5). The adult female gray (ID# 1100) whale's baleen had significantly higher progesterone than the juvenile (ID# 1019, 1025) whales (33.9 ± 15.1 ng/g lipid, ANOVA, $F = 18.4$, $p = 5.5$ x 10-5). The adult male humpback (ID# 1024) whale's baleen had significantly higher testosterone than the juvenile (ID# 1015) whales' baleen (52.6 ± 101.6 pg/g lipid, ANOVA, $F = 7.3$, $p = 0.009$) (Table 2).

Earplug. Cortisol concentrations for earplugs showed that the adult female gray whale (ID# 1100) and adult male humpback whale (ID# 1024) had significantly higher cortisol concentrations than juvenile whales (9.6 ± 3.6 and 5.7 ± 1.5 ng/g lipid, respectively, ANOVA, $F = 97.6$, $p < 2$ x 10-16). The adult female gray (ID# 1100) whale's earplug had significantly higher progesterone than the juvenile (ID# 1019, 1025) whales (10.6 ± 5.0 ng/g lipid, ANOVA, $F = 443.1$, $p < 2$ x 10-16). The adult male humpback (ID# 1024) whale's earplug had significantly higher testosterone than the juvenile (ID# 1015) whales' earplug (1.8 ± 1.2 pg/g lipid, ANOVA, $F = 7.5$, $p = 0.009$) (Table 3).

Relationship among blubber, baleen, and earplugs

Comparing the corresponding cortisol, progesterone, and testosterone Z-scores in the earplug and baleen reveals a positive correlation (Pearson's product moment correlation, $r2 = 0.43$, $t = 16.8$, $p = 0.019$, Fig. 2), though a similar exploration between baleen and blubber exhibited no significant relationship.

To ascertain how hormone concentrations in three biological matrices relate, the earplug and baleen hormone concentrations from samples representative of the most recent time frame were compared to full thickness blubber hormone concentrations and to one another. After the removal of one outlier, cortisol measurements for whale ID #1100 (earplug cortisol was a significant outlier, Grubbs' test, $p = 0.02$, blubber cortisol was significant outlier, Grubbs' test, $p = 0.001$), a significant positive correlation was found between the most recent earplug cortisol or reproductive hormone concentration (estimation of the last six months of life) of a single lamina and most recent baleen hormone concentration (estimation of the last few weeks of life) of a single sample from each individual (Pearson's product moment correlation, $r2 = 0.77$, $t = 3.2$, $p = 0.02$). A significant positive

correlation was also found between the cortisol and reproductive hormone concentrations of the most recent baleen samples and full blubber thickness samples (representative of the last days to months of the whale's life) (Pearson's product moment correlation, r2 = 0.97, t = 10.3, p = 1.7 x 10^-5). A significant positive correlation was also found between the cortisol and reproductive hormone concentrations in the most recent earplug laminae to full blubber thickness samples (Pearson's product moment correlation, r2 = 0.77, t = 3.2, p = 0.01) (Table 3).

C. Contaminants in baleen whale earplugs

Chemicals, supplies, extraction, and quantification were performed as previously described by Robinson et. al., (2013). The complete target list contains 53 analytes from 3 major POP groups including, pesticides, PCBs, and polybrominated diphenyl ethers). ?DDXs represents the total of both isomers (i.e., p,p' and o,p') of DDT, DDE, and DDD. ?DDT, ?DDE, and ?DDD represent the total of both isomer (p,p' and o,p') of DDT, DDE, and DDD, respectively. ?PCBs and ?PBDE include the total of all quantified congeners for each specific class of POPs, while ?CHLR including cis- and trans-chlordanes, and ?NCHL includes cis- and trans-nonaclor. Due to matrix interference, the DDT analysis (including both isomers, o,p', and p,p', of DDT, dichlorodiphenyldichloroethylene (DDE), and dichlorodiphenyldichloroethane (DDD)) was performed using gas chromatography-tandem mass spectrometry in electron ionization mode (GC-MS/MS EI).

Earplugs were delaminated, aged, and stored as with protocol developed in our labs. Earplugs were received from five fin whales (n = 5), three adults from archived collections and two juveniles from recent stranding. The lifespan (1.5 to 31 y) of the whales, based on aged earplugs, spanned 1928-2016 (~87 y). Individual whales identified by species as well as ocean basin of origin (e.g. NA or NP for North Atlantic or North Pacific, respectively) and lifespan; see Table 4. Single laminae were analyzed for POPs when sample mass was adequate (i.e. 150 mg), however, when sample mass was insufficient, adjacent laminae were combined as needed. A total of 35 earwax sample extractions (n=35) were used to characterize the life history of five fin whales (Table 4). All POP concentrations were reported as ng g-1 and were corrected for the mass of earwax (g) used in the extraction. The average age and calendar year of each extraction was determined by averaging the age or year of each lamina included in the sample extraction. For example, if laminae 8-15 were combined, this would correspond to age 4-7.5 for which the average age would be 5.8 years. For each earplug, the species, sex, lifespan, estimated age, and ocean of origin can be found in Table 4.

Using modern analytical extraction systems and instrumentation (e.g., SPLE and GC-MS), we were able to extract and analyze POPs that have been archived within lamina (i.e. 1930) including both isomers (p,p'- and o,p'- isomers of DDX). POP exposure profiles was determined from earplugs up to 40 years earlier than previously published marine mammal POP studies.1 Three fin whale earplugs received spanned the 1920s to early 1960s; NA1, NA2, and NP1 (Table 4). The remaining three earplugs, included one blue whale (NP2, previously described by Trumble et al., 2013) and two fin whales, spanning two decades from 1995 to 2015 (i.e. NP2, NP3, and NP4).

Three whales (NA1, NA2, and NP3) were used to investigate the usage periods of DDT spanning the 1930s to the 1960s. The relative contribution to percent composition (over the past eight decades) of all POP classes or individual compounds of all six baleen whale earplugs were ?DDXs (95%) >> ?PCBs (3%) > ?PBDEs, ?CHLRs, ?NCHLRs, HCB, PCA, and ?-HCH (< 1% contribution); (Fig. 3). Similarly, ?DDX (reporting only p,p' isomers of DDT, DDE, and DDD), ?PCBs, ?-chlordane, and Dieldrin measured in the blubber of humpback whales within the North Atlantic ocean revealed concentrations of ?DDX greater than ?PCBs.18 Both isomers of DDT, as well as metabolites (DDE and DDD), were detected within the selected fin whales as early as the 1930s (Fig. 4A-C). NP1 (male fin whale, 1928-1961) temporally coincided with the widespread use of DDT and had a rate of increase approximately 10 ng·g-1 y-1 from 1928 (i.e., it's birth) to 1935.

?DDX measurements dated back to 1935 from earplugs reached concentrations of approximately 1,200 ng g-1 in NP1, which was 63 times that of DDX measured in NA1 (19 ng g-1, 1937) and 210 times that of NA2 (5.6 ng g-1, 1938). Interestingly, NP2, a male blue whale living from 1995-2007, was also dominated by DDX, consisting mainly of metabolites, and suggest that DDX was a major contributor to POP burden decades after restricted use and could potentially result in an elevated risk scenario for North Pacific baleen whales in comparison to the North Atlantic. NP2 reconstructed profile also showed a significant fraction of the POP burden occurred in the first lamina, which may be described by the transfer of lipophilic compounds from mother to offspring (i.e., maternal offloading).

To assess maternal offloading further, earplugs from NP3 and NP4 were both selected due to their overlapping lifespan, age, ocean of origin, and high sample mass per lamina. Reconstructed POP profiles from NP3 were dominated by DDX with 5.4 times higher POP burden within the first lamina than NP4. This domination by DDX was like the profiles measured in NA1, NA2, and NP1 (lifespans that included heavy POP usage periods). Interestingly, NP4 revealed a more modern profile dominated by HCB and ?CHLRs, followed by ?DDXs and ?PCBs, which was different than the other 5 profiles. This difference in further pronounces within the DDX profile (Fig. 4E and 4F). For example, NP3's first lamina contained all quantified isomers of DDX (p,p'- and o,p'- DDT, DDE, and DDD). NP4's first lamina contained both isomers of DDT and DDD only.

Further differences were found between the first and subsequent laminae within DDX profiles. As mentioned above, the first lamina of NP3 contained all quantified isomers of DDX, however, the following laminae only contained DDE and DDD (Fig. 4E). NP4's subsequent layers were also different from the first as p,p'- DDT was only detected within the first lamina (Fig. 4F), which suggests differences between maternal and environmental exposure. Interestingly, POP profiles varied between individuals and laminae within the same ocean basin and time span (2013-2015), which suggest variations in maternal POP burden as well as regional difference (e.g., feeding grounds).

Time-series data reconstructed from an individual whale can be evaluated as a function of year and age (bioaccumulation rate sections). Multiple reconstructed DDX and PCB profiles (1928-2015; n=4) were combined to illuminate the historical exposure of marine organisms as a function of year within the Pacific Ocean (Fig. 5). This nearly 90-year record closely matches historic-use of DDT and PCBs in the Northern Hemisphere. Interestingly, DDX concentrations are higher than PCBs, which is supported by the tonnage used and application practice between pesticides (released directly into the environment) and industrial applications (PCBs). It is estimated that only about 30% of PCBs released into the environment have reached the oceans. As expected, concentration of DDX, and PCBs were highest during periods of use and then subsequently decreased after restrictions. Within baleen whales, DDX and PCB concentrations increased from the 1930s to early 1950s and were relatively stable during the 1950s and 1960s. Breivik et. al., (2016) modeled PCB concentrations reaching a maximum in the environment during the late 1970s, however laminae analyzed in this study did not span this period. Beginning in 1995, the profile indicated a reduction in concentration three decades after been heavily restricted. With additional earplugs, it is possible to further elucidate the strength of the model and fill knowledge gaps of open ocean systems during usage periods.

With time series data we can evaluate how legacy POPs move from one generation to the next. Within the DDX and PCBs model (Fig. 5), two points were outliers beyond the predicted curve. These outliers, circled in green, belong to the first lamina of NP2. The subsequent laminae (n = 23) provide the largest contribution to the model following the restrictions placed on DDT and PCBs. The first lamina was, on average, 5 times higher in POP concentrations than the subsequent laminae. Interestingly, NP3 and NP4 did not deviate as drastically from the exposure model. Using time series data, we can capture the environmental exposure from previous generation (i.e., maternal offloading is indicative of the environmental exposure of the mother). The first lamina in NP2 suggest that the mother had a large POP burden and likely lived during the during or closely after usage was heavily restricted. Alternatively, NP3 and NP4's first lamina did not contain dramatic difference and indicate less generational difference between mother and offspring in comparison to NP2. POP exposure is dependent not only on the lifespan of the individual, but also the lifespan and exposure from their mother and can be differentiated using baleen whale earplugs.

Bioaccumulation Rates

Life-time bioaccumulation rates (specifically burden as a function of age; ng.g-1 y-1) help correct for the difference in age between individuals. This correction improving our assessment of key scientific questions regarding temporal and spatial differences in POP burdens. Lifetime bioaccumulation rates were calculated using linear regression (Fig. 6) and are extremely rare, especially for free-ranging long-lived marine mammals. To the authors' knowledge, bioaccumulation rates in marine mammals have only previously been modeled or assessed in captive beluga whales.

Age-corrected earplug data was used to assess spatial differences between the Pacific and Atlantic Oceans. Bioaccumulation rates ranged from 2.6 to 470 ng·g-1 y-1, had coefficients of determination (r2) ranged from 0.86 to 0.99. The analysis of the bioaccumulation rates revealed that the bioaccumulation rates in the North Pacific (n=4) were 56

times higher than those measured in the North Atlantic (n=2). NP1 had the largest at a rate of 470 ng·g-1 y-1, followed by NP2 (360 ng·g-1 y-1), NP3 (95 ng·g-1 y-1), and NP4 (43 ng·g-1 y-1). The bioaccumulation rates measured in the North Atlantic were 5.9 and 2.7 ng·g-1 y-1 for NA1 and NA2, respectively.

The overarching assumption is that the rate of increase in bioaccumulation (i.e., slope) would be proportional to the bioavailable fraction of POP within an ecosystem. Multiple regression analysis and analysis of covariance (ANCOVA) were performed with JMP 14.0 (SAS Institute, Cary, NC) to determine if the slopes were statistically different between individuals ($p < 0.05$). The model consisted of age and ID as the independent variables, burden as the dependent variable. Average age and ID were determined to be significant ($p = 0.0004$ and < 0.0001, respectively) and were then crossed within the model resulting in a significant interaction ($p < 0.0001$) indicating a significant difference between slopes. The difference in bioaccumulation rates between slopes may highlight the regional difference in POP contamination, which is an important aspect for assessing differences in cetaceans and other marine organism stress and health.

When evaluating the POP concentrations over the lifetime of an individual it is important to consider the transfer of historic POPs from one generation to the next as well as the impact during early developmental periods and chronic exposure. Previous studies of marine mammals have found that increased POP burdens have led to immunosuppression in calves (increased susceptibility to disease) and could also lead to immunotoxicity, cancer, and endocrine disruption. Contaminants derived from the environment (e.g., diet and location) are also important for long-lived species. For example, bioaccumulation rates indicate a difference (concentrations and profiles) between different regions of the world (e.g., North Pacific and Atlantic Ocean Basin). It is important to consider both generational transfer and environmental exposure when considering the health and management of marine species.

D. Bulk stable isotopes recovered from baleen whale earplugs

Using time-series datasets from biological tissues can provide us a great opportunity to reconstruct past ecosystems and assess how individuals and populations respond to the changing environment through time. Carbon (?13C) and nitrogen (?15N) stable isotope datasets have been used to determine foraging location and trophic status of organism higher at the trophic lever, due to the predictable change through the trophic level relative to the prey. In addition, in marine ecosystem, ?13C and ?15N values at the base of the food web (i.e. baseline) vary both latitudinally as well as along inshore/offshore gradients to create a spatially distinct isotopic value, known as isoscapes, that can be used to track movement pattern and trophic interactions of organisms.

Increasing human land and ocean-based activities accompanied by anthropogenic climate change have been impacted key hydrological and physiochemical processes in ocean ecosystem such as sea surface temperature (SST), turbidity, wind, stratification, and nutrient concentration. Variability in oceanographic parameters could alter primary productivity at the base of the food web and force individuals higher at the trophic level to temporarily move outside their usual foraging habitat or feed on a different trophic level and subsequently change population spatial dynamic and trophic interactions. Additionally, emission of depleted 13CO2 from fossil fuel combustion, referred to as the Suess effect, ultimately dissolves in oceans and resulted in reduction of isotopic composition of baseline through time due to preferential uptake of 12C by phytoplankton.

Baleen whales are long-lived marine mammals with annual long-distance migrating between high-latitude summer grounds and low-latitude winter grounds. Known as marine sentinels, variation in whale feeding, foraging, and migration could be indicative of marine ecosystem health. Thus, reconstruction of time-series ?13C and ?15N datasets of marine organisms such as baleen whales can serve to examine cause-and-effect relationships involving aspects of behavior and ecosystem-level changes in regional and global scale. Interestingly, baleen whales have earplugs, a keratin-rich matrix capable of recording and archiving the life-history of an individual whale with six-months resolution. Reconstruction of lifetime ?13C and ?15N profiles (i.e. birth to death) using earplug would enable us to investigate years to decadal change in both ocean ecosystem and individual foraging ecology.

The purpose of this study is to analyze ?13C and ?15N values of baleen whale earplugs with the potential to provides an unprecedented chance to reconstruct life history foraging ecology of baleen whales. Additionally, utilize longitudinal stable isotope profiles to assess behavioral changes (e.g., shift in foraging location or trophic position) of baleen whales in response to the changing as well as environment ecosystem-level changes (i.e. the Suess effect and variation in baseline isotopic values as well as change in

biogeochemical cycles) over a large time-and space-scale.

Stable Isotope Analysis
Approximately 1 mg of homogenized laminae were analyzed for ?13C and ?15N composition in the Baylor University stable isotope laboratory using a continues flow elemental analyzer coupled with an isotope ratio mass spectrometer. Stable isotope ratios are presented in delta (?) notation in parts per thousand (‰) as defined by the following equation:
?X=(((R_Sample)/R_Standard)-1)*100 (1)

Where X is 13C or 15N, and R corresponds to the 13C/12C or 15N/14N ratio of the samples and standards. Vienna Pee Dee Belemnite (VPDB) calcium carbonate and atmospheric nitrogen were used as standards for 13C and 15N, respectively. ?13C values were corrected for earwax lipid composition using the modified McConnaughey and McRoy (1979) model as described previously.

Assessing lifetime stable isotope profiles
Whales annually migrate between higher latitudes foraging grounds and lower latitudes breeding grounds, however, they may forage to a different region from year to year or switch between prey items from different trophic level which could result in an increase or decrease in ?13C and ?15N composition of their tissues. For example, if an individual consistently migrates between two isoscapes and feed on the same trophic level, the long-term profile should be relatively consistent with a small standard deviation. However, if an individual move to a different isoscapes or feed on a different trophic level, it can be resulted in short-term change (i.e. 1-4 yrs) or gradual change (i.e. over 5 years) in ?13C and ?15N values. In this study, short-term changes in ?13C and/or ?15N values were identified as behavioral events using the standards signal-to-noise ratio (S/N) of 3:1. Here, the signal represents the magnitude of change in stable isotope values, while the noise represents the background stable isotope value. Moreover, variability in bulk stable isotope values in baleen whales was measured using standard deviation across all individual's laminae.

Suess Effect Correction
To eliminate impact of excessive depleted 13CO2 input into the atmosphere and marine system and provide a comparable ?13C values between recent and historical samples, ?13C time-dependent values of earplugs were corrected for the Suess effect using Hilton et al (2006) equation:
Suess effect correction factor =a*?exp?^((b*0.027)) (2)
Where a is the annual rate of ?13C decrease for the water body and b is the assigned year for laminae minus 1850 (i.e. onset of the industrial revolution and mass fossil fuel burning). Since rate of ?13C depletion of dissolved inorganic carbon (DIC) varies globally due to the physiochemical properties of water, -0.014‰ and -0.018‰ were used for the annual rate of ?13C depletion in the North Pacific and the North Atlantic Ocean, respectively. Since the contribution of atmospheric CO2 associated with burning fossil fuels varies as a function of time, the Suess effect correction factor specifically calculated for each year and were added to the ?13C values of corresponding laminae. For example, ?13C values of laminae from 2010 were adjusted by +1.05 and those from 1950 were adjusted by +0.27.

Furthermore, temporal trends in ?13C profiles, associated with known source anthropogenic Suess effect, were identified using simple linear regression using SPSS Statistics v.22 (IBM Corporation, NY, USA).

Results
?13C and ?15N values of all laminae (n = 337) were successfully measured. ?13C values were corrected for lipid composition using a normalized lipid correction model. Then, ?13C and ?15N values were normalized to their corresponding year and age and were used to reconstructed lifetime ?13C and ?15N profiles in baleen whales with six-month resolution (Fig. 7).

Within all six individuals, mean ± standard deviation (SD) for ?13C values ranged from -25.81 ± 0.14‰ to -16.80 ± 0.38‰ (Table 1). Among the Northeast Atlantic (NEA) whales, fin whales EP006 and EP016 showed higher ?13C value (-20.06 ± 0.16‰ and -20.54 ± 0.26), while lower ?13C values were found for two blue whales: EP041 and EP040 (-25.81 ± 0.14‰ and -25.69 ± 0.18‰, respectively). Among the Northeast Pacific (NEP) whales, humpback EP027 demonstrated higher ?13C value (-16.80 ± 0.38‰) followed by EP022 (-19.55 ± 0.83‰).
The mean ± SD for ?15N values ranged from 6.02 ± 0.25‰ to 13.69 ± 0.73‰ among six individuals. Within the NEA whales, mean values of ?15N ranged from a low of 6.02 ± 0.25‰ for a blue EP040 to a high of 10.74 ± 0.34‰ for fin EP016 (Table 6). Among the NEP whales, mean values of ?15N ranged from a low of 10.91 ± 0.27‰ for the humpback EP027 to a high of 15.72 ± 0.73‰ for the humpback EP022 (Table 6).

Significant enrichment and depletion events were identified (S/N 3:1) in ?13C and ?15N values over relatively short period of time (i.e. 1-4 yrs) of an individual's life. For example, events were identified in ?13C and ?15N of blue whales EP040 and EP041 (Fig. 7a,b). ?13C values of blue whale EP040 decreased from -25.80‰ in 1950 to -26.28‰ in 1951 (S/N = 5.3), increase again to -25.86‰ a year after. In addition, ?15N of blue whale EP041 increased from 5.87‰ in 1937 to 7.14‰ in 1938 (S/N = 7.9) and back down again to again to 5.82‰ (Fig. 7a,b).
Interestingly, variability observed in ?13C profile from humpback whale EP022 varies across individual's life. Variability in ?13C composition of earplug from the 1980s to 2000s (SD = ± 1.17) was higher when compared to variability in ?13C values of earlier periods of the individual's lifespan (SD = ± 0.47) (Fig. 8c). For example, from 1986 to 1992 there was 4.56‰ shift in ?13C values (-17.73‰ to -22.29‰; ± 1.45) as compared to 1945 to 1980 era which demonstrated 2.29‰ change in ?13C value (-18.43‰ to -20.72‰; ± 0.50). Like ?13C values, humpback EP022 displayed higher variabilities in ?15N values later in life (Fig. 8d). For example, ?15N values decrease from 14.84‰ to 12.48‰ during 1993 to 1996 and increase again from 12.56‰ to 15.72‰ between 1998 and 2000 (Fig. 8d), while this individual experienced relatively small changes in ?15N values during 1964 to 1968 (13.19‰ to 14.63‰). Additionally, period of variabilities was observed in ?15N profiles of humpback whale EP027. For example, ?15N values of earwax increased from 12.77‰ in 1958 to 14.12‰ in 1963 and decreased from 14.14‰ in 1965 to 12.68‰ in 1969.

In addition to behavior changes, long-term trends (>10 yrs) were identified using a simple linear regression model. Interestingly, five out of six individuals from both Atlantic and Pacific Oceans demonstrated long-term significant negative trend in ?13C values ($p < 0.05$). The overall slopes ranged from -0.035 ± 0.003‰ yr-1 to -0.016 ± 0.003‰ yr-1 with R2 ranging from 0.45 to 0.73 ($p < 0.05$) (Table 6, Fig. 8). However, no long-term trends were identified within six individuals' ?15N profiles.

Furthermore, ?13C values were evaluated for the known source of ?13C depletion in the marine ecosystem (i.e. Suess effect) using the equation above (Eq. 2). Afterward, five individuals' ?13C profiles were revaluated and long-term significant declining trend were observed in ?13C values of all five individuals. The overall slopes for the Suess corrected ?13C profiles ranged from -0.027 ± 0.003‰ yr-1 to -0.010 ± 0.002‰ yr-1 with R2 ranging from 0.23 to 0.63 ($p < 0.05$) (Fig. 8). Result of statistical comparison of slopes before and after the Suess correction showed that there is a significant difference between slope of raw and the Suess corrected ?13C values in 1016 (fin, $t = 2.80$, $df = 124$, $p < 0.05$) and 1027 (humpback, $t = 1.99$, $df = 120$, $p < 0.05$). However, no significant difference was observed between raw and the Suess corrected slopes of 1006 (fin, $t = 0.73$, $df = 44$, $p > 0.05$), 1040 (blue, $t = 2.80$, $df = 124$, $p > 0.05$), and 1041 (blue, $t = 2.80$, $df = 124$, $p > 0.05$). Our result revealed that known source of global decline in ?13C value of marine ecosystem, explained only 1.33 ± 0.12% of the decrease in ?13C values of EP006, 1.38 ± 0.33 % of EP016, 0.93 ± 0.13% of EP040, 0.89 ± 0.14% of EP041, and 1.69 ± 0.38% of EP027.

(See equations, tables, and figures attached).

Description: Sample sources may include:
- Animals in foreign countries stranded alive or dead or that died during rehabilitation;
- Animals killed during legal subsistence harvests;
- Animals killed incidental to legal commercial fishing operations; or
- Samples from other authorized persons or collections (e.g. museums).

The number of animals (by species and taxa group), life stage, and sex are outlined in the take table. This study does not include the receipt, development or use of cell lines.

Types of Samples
Parts may include any hard or soft part from marine mammals; however, we expect the majority of our samples to consist of earplugs, baleen, and teeth. Most earplugs and corresponding baleen will be sampled from several museums including the Smithsonian Institute, Nature Museum of Canada, Natural History Museum of London, and University of Barcelona and have been granted partial access to their samples. These samples will be imported through a designed USFWS port of entry (likely DFW). Samples from these species have been acquired during previous whaling activities. While not available for all samples, many samples held in museums do describe original take and location. We will make every attempt to ensure samples used during this study were not collected from illegal sources.

Most samples have been collected and stored in museum holdings and universities (and not all fully catalogued). Not fully catalogued refers to not having individual whale identification/data available. For example, in the Smithsonian, the Dale Rice collection from the 1960s contains several hundred gray whale earplugs. Many of these plugs were stuffed into large glass jars with only Date and Species (1966; E. robustus) and while we may be able to use these plugs for larger questions (population stress) there are other questions (sex-based, aging) that cannot be answered. When available, we will verify earplug or baleen samples location and year of collection to ensure collection was not in violation of the MMPA and only from sanctioned or authorized whaling events (i.e. we will not accept samples taken from current whaling activities collected from Japan, Russia, etc). We are not sampling from live animals. Any samples that were collected prior to the MMPA (1972) or the ESA (1973) will be received under a separate authorization (i.e., Letter of Determination). Any samples that are post-act would be received, imported, or exported under this scientific research permit.

Earplugs/baleen collected and housed in the U.K. and Spain were collected during whaling activities in the northern and southern hemispheres between 1909-1955 (UK) and 1951-1985 Spain. Samples in Spain were collected during the last two whaling stations in NW Spain (Galicia): Caneliñas (located in Cee, province of La Coruña), active between 1951 and 1985 (last year of whaling in Spain) and Balea (Cangas, province of Pontevedra), with activity between 1955 and 1983.
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For subsistence hunted animals, we will verify collection was not in violation of the MMPA and only from legal, sanctioned, or authorized subsistence events to the best of our ability. We will make every attempt to ensure samples used during this study were not collected from illegal sources.

We may also receive parts from U.S. stranded marine mammals under separate authorization letters. These parts may be exported and re-imported for analysis or curation with our collaborators.

Sample preservation and transport. Baleen whale earplugs sampled from stranded species will be stored and shipped cold (dry ice or ice packs) overnight (FedEX) to Baylor University. Received samples will be stored in an ultra-cold freezer (-80C) until processed. Earplugs from archived museum holdings will be wrapped in cheesecloth and aluminum foil and placed in plastic Ziplock bags and shipped and stored as mentioned above.

Baleen is typically shipped cold overnight in a cooler and when received, the baleen is thoroughly cleaned and dried and stored dry in a plastic bag in a positive pressure room.

Analytical techniques

Stress-related hormone radioimmunoassay technique

During the extraction phase of an earplug or baleen, a minimum of 15 mg of each lamina layer will be added to 2 ml of diethyl ether, vortexed for 30 seconds and subsequently centrifuged for 30 seconds. A series of experiments determined that 15 mg was the minimum amount of lamina wax needed to produce consistent results. The resulting supernatant will be pipetted into a separate vial and stored. This process will be repeated twice in which time the supernatant will be placed under a nitrogen stream until dryness, capped and stored in a 4 °C refrigerator covered in foil up to 1 week. The waxy pellet will be suspended sing 250 µl of assay buffer (from ELISA kit) into vial and vortexed for 30 seconds. Cortisol extractions will be assayed using a competitive immunoassay kit (Enzo Life Sciences; 900-071) with validation and parallelism techniques applied. Each extraction will be run in duplicate on a 96-well microtiter plate (Beckman Coulter DTX 880 Multimode Detector). The cortisol in the sample binds competitively with the enzyme conjugated to cortisol. An added substrate detects enzyme activity resulting in an inverse relationship between optical density and the amount of cortisol in the sample. This inverse relationship will be calculated using a standard curve with results converted to ng/g.

Contaminant extraction methodology

POPs will be extracted from cerumen homogenates using selective pressurized liquid extraction (SPLE), which combines pressurized liquid extraction (PLE) with adsorbent cleanup techniques into a single automated technique. SPLE will be performed using an accelerated solvent extractor (ASE 350, Dionex, Salt Lake City, UT) with 66 mL ASE extraction cells. The final method will consist of homogenization of an aliquot of whale cerumen (~0.25 g) with sodium sulfate (baked at 500 °C for 12 hours and allowed to cool) utilizing a mortar and pestle. Cerumen homogenates will be placed on top of pre-cleaned sorbents (with an order of basic alumina oxide, silica gel and florisil from top to bottom) within the ASE cell. The sorbents will be pre-cleaned using 1:1 (v/v) dichloromethane (DCM):hexane (HEX) under the following ASE conditions: 100 °C, 1500 psi, and 50% rinse volume. Cerumen homogenates will be spiked with isotopically labeled surrogate standards to correct for target analyte loss during sample preparation and will be allowed to come to equilibrium for 1 hr prior to extraction. Next, cerumen homogenates will be extracted with DCM:HEX (1:1) under the same ASE conditions as described above except with a 150% rinse volume. ASE extracts will be concentrated to ~0.3 mL using a Turbo Vap II from Caliper (Hopkinton, MA), then transferred to a gas chromatography (GC) vial and spiked with isotopically labeled internal standards prior to analysis.

Contaminant Extract Analyses

The analysis of pesticides, PCBs, and PBDEs will be performed using a 7890 gas chromatograph coupled to a 5975 mass spectrometer (Agilent Technologies, Santa Clara, CA) in electron capture negative ionization (ECNI) or electron impact (EI) with selective ion monitoring. One microliter of sample extract will be injected utilizing an Agilent 7683 Injector in a pulsed splitless mode (pulse at 20 psi until 0.74 minutes). The injection port will be set to 300° C. Chromatographic separation will be achieved using a DB-5 capillary column (J&W, 30 m x 0.25 mm i.d.; 0.25µm film thickness). All analytes except for p,p'-DDT, p,p'-DDE, and o,p'-DDE will use an oven temperature program of 120 °C, held for 1 min, ramped to 275 °C at 4 °C min-1, then ramped to 320 °C at 6 °C min-1, and held for the final 5 minutes. The total run time will be 52.25 minutes. Helium (99.999%) will be used as the carrier gas, and methane (99.999%) will be the buffer gas. The ECNI ion source and quadrupole mass analyzer temperatures will both be set to 150 °C.

p,p'-DDT, p,p'-DDE, and o,p'-DDE will be analyzed using the same instrumentation and parameters as described above except in EI mode with an oven temperature program of 120 °C for 1 minute, and then ramped at 4 °C min-1 to 250 °C for a total run time of 33.5 minutes and a source temperature of 230 °C. The quadrupole mass analyzer temperature will be 150 °C.

Quality Assurance and Quality Control (QA/QC)

Target analytes will be identified based on retention times (±0.05 min) as well as a quantitative to qualitative ion response ratio (±20%). Ion responses ratios will be based on continuous calibration verification standards analyzed prior to sample analysis. All target analytes will be identified using a single quantitative ion and two qualitative ions,

except for p,p'- DDT, which has one quantitative ion and one qualitative ion. Target analyte concentrations will be determined using a calibration curve with at least seven points ranging several orders of magnitude. Target analyte calibration curves will plot the response dependent concentration factor of the target analyte (concentration of target analyte divided by the concentration of its surrogate standard) versus the concentration dependent response factor of the surrogate standard (response of the target analyte divided by the response of its surrogate standard). Target analyte calibration curves will be linear and forced through the origin and have coefficients of determination (r2) of at least 0.99. Surrogate recoveries will be quantified using internals standards spike prior to analysis.

Calibration curve check standards will be run before and after each sample batch to validate the integrity of the calibration curve. Calibration curves and calibration check standards will be prepared simultaneously. Calibration check standards concentrations will correspond to the upper-middle point of the calibration curve. Calibration of standard concentrations not within ±30% of the prepared concentration will require instrument maintenance and subsequent re-running of the sample batch.

Supplemental Information

Status of Species:	The species included in the samples are as follows (all marine mammals protected by MMPA); grey whale (E. robustus; Western North Pacific ESA, endangered; MMPA, Depleted; CITES, Appendix I; Eastern North Pacific, not listed), blue whale (B. musculus; ESA, Endangered; MMPA, Depleted; CITES, Appendix II), fin whale (B. physalus; ESA, Endangered; MMPA, Depleted; CITES, Appendix I), humpback whale (M. novaeangliae; ESA, Threatened to Endangered; MMPA, Depleted; CITES, Appendix I), bowhead whale (Balaena mysticetus; ESA, Endangered; MMPA, Depleted; CITES, Appendix II), sperm whale (Physeter macrocephalus; ESA, Endangered; MMPA, Depleted; CITES, Appendix II), minke whale (Balaenoptera acutorostrata; MMPA, Protected; CITES, Appendix I), Sei whale (Balaenoptera borealis; MMPA, Protected, CITES, Appendix I), Bryde's whale (B. brydei; MMPA, Protected; CITES, Appendix II), Rice's whale (B. ricei; ESA, Endangered; CITES, Appendix I, MMPA, Protected and depleted), right whales (including N. Pacific, N. Atlantic, S. Right; Eubalaena spp.; MMPA, Protected; CITES, Appendix I, MMPA Depleted).
Intentional Lethal Take:	Not Applicable; request is for archived or previously collected marine mammal parts; no live animals will be affected or killed for the purposes of providing samples for our research. Samples will also be acquired from stranding networks/necropsies, fisheries bycatch, or legal subsistence harvests.
Anticipated Effects on Animals:	Not Applicable; request is for marine mammal parts/tissues from dead animals including: stranded (deceased) animals in foreign countries, museums, fisheries bycatch, or legal subsistence harvests, no live animals will be affected for our research activities.
Measures to Minimize Effects:	Not Applicable, no negative effects on animals; request is for marine mammal parts, no live animals will be affected for our research activities.
Resources Needed to Accomplish Objectives:	Access to samples has been granted in several museums and U. of Barcelona. Earplugs are assessed for structural integrity (sometimes they are too dry to work with) and cut into halves (leaving ½ to the museum etc). The amount of sample is up to the discretion of the museum or university.
Disposition of Tissues:	Samples will be destroyed in analysis. However, this is not to taken lightly, for we realize these plugs are irreplaceable. Some analysis can be used without destroying the plugs; radio isotopes and elemental analysis, however, aliquots of cerumen are used for all contaminant and hormone analysis. Since there is such small mass per lamina there is typically nothing remaining after analysis. It should be mentioned that for the bowhead earplug we recovered, stable isotopes, 50 contaminants, a suite of hormones, metals, and cesium and lead. These parts may be exported and re-imported for analysis or curation with our collaborators.

Public Availability of Product/Publications: The result of our research are made available to the public through scientific journals, presentations at scientific meetings, and public media stories and articles. Some examples of publications and media coverage include:
1. The surprising science of whale earwax/live talk with NHM scientist. 2020, www.youtube.com/watch?v=IaL8eaGcFlQ
2. What can we learn from whales' earplugs? | Natural History Museum; 2019.
3. Nature Research Ecology and Evolution Community, 17 October 2018: invited to post a 500 word blog on this Nature website. https://natureecoevocommunity.nature.com/
4. Swiss Public Radio www.srf.ch/play/radio/wissenschaftsmagazin/audio/leben-aus-dem-baukasten?id=dc33c243-87bb-4b2d-a67c-094e937acbbc&startTime=1150
5. The Guardian, Sept. 17, 2013
See attached for complete li

Location/Take Information

Location

Research Area: Animal Parts

Location Description: Smithsonian Institute, Natural History Museum London, Natural History Museum Santa Barbara, Natural History Museum Los Angeles County, University of Barcelona (Spain). Canadian Museum of Nature (Ottawa)

Take Information

Line	Ver	Species	Listing Unit/Stock	Production /Origin	Life Stage	Sex	Expected Take	Takes Per Animal	Take Action	Observe /Collect Method	Procedure	Transport Record	Begin Date	End Date
1		Whale, blue	Range-wide (NMFS Endangered)	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs and baleen												
2		Whale, bowhead	Range-wide (NMFS Endangered)	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs and baleen												
3		Whale, Bryde's	Range-wide	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs and baleen												
4		Whale, fin	Range-wide (NMFS Endangered)	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs and baleen												

5		Whale, gray	Range-wide	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs and baleen												
6		Whale, humpback	Range-wide (NMFS Endangered)	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs and baleen												
7		Whale, minke	Range-wide	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs and baleen												
8		Whale, Rice's	Range-wide (NMFS Endangered)	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs and baleen												
9		Whale, right, North Atlantic	Range-wide (NMFS Endangered)	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs and baleen												
10		Whale, right, North Pacific	Range-wide (NMFS Endangered)	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs and baleen												
11		Whale, right, southern	Range-wide (NMFS Endangered)	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs and baleen												
12		Whale, sei	Range-wide (NMFS Endangered)	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs and baleen												
13		Whale, sperm	Range-wide (NMFS Endangered)	Wild	All	Male and Female	100	1	Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026

		Details: hard and soft parts, including earplugs and teeth												
14		Cetacean, unidentified	NA	Wild	All	Male and Female	100		Import/export/receive only	Other	Export; Import; Receive domestically	N/A	11/23/2021	11/30/2026
		Details: hard and soft parts, including earplugs, baleen, and teeth from species not specified above												

NEPA Checklist

1) If your activities will involve equipment (e.g., scientific instruments) or techniques that are new, untested,or otherwise have unknown or uncertain impacts on the biological or physical environment , please discuss the degree to which they are likely to be adopted by others for similar activities or applied more broadly.

Not applicable; no methods are new or untested. In a museum setting we use ceramic knives for sectioning earplugs and a Dremel tool for drilling baleen.

2) If your activities involve collecting, handling, or transporting potentially infectious agents or pathogens (e.g., biological specimens such as live animals or blood), or using or transporting hazardous substances (e.g., toxic chemicals), provide a description of the protocols you will use to ensure public health and human safety are not adversely affected, such as by spread of zoonotic diseases or contamination of food or water supplies.

Not applicable; earplugs and baleen are inert tissue and have never been documented to be associated with disease or disease outbreaks.

3) Describe the physical characteristics of your project location, including whether you will be working in or near unique geographic areas such as state or National Marine Sanctuaries, Marine Protected Areas, Parks or Wilderness Areas, Wildlife Refuges, Wild and Scenic Rivers, designated Critical Habitat for endangered or threatened species, Essential Fish Habitat, etc. Discuss how your activities could impact the physical environment, such as by direct alteration of substrate during use of bottom trawls, setting nets, anchoring vessels or buoys, erecting blinds or other structures, or ingress and egress of researchers, and measures you will take to minimize these impacts.

Not applicable; we will be working in a museum and/or laboratory setting. For example, at the London Museum of Natural History, we worked in the basement lab in a positive pressure environment.

4) Briefly describe important scientific, cultural, or historic resources (e.g., archeological resources, animals used for subsistence, sites listed in or eligible for listing in the National Register of Historic Places) in your project area and discuss measures you will take to ensure your work does not cause loss or destruction of such resources. If your activity will target marine mammals in Alaska or Washington, discuss measures you will take to ensure your project does not adversely affect the availability (e.g., distribution, abundance) or suitability (e.g., food safety) of these animals for subsistence uses.

Not applicable; while we do have access to Bowhead whale earplugs from Alaska, the plugs are collected during the processing of the whale by the local native Alaskans. Gray whale earplugs have also been collected from Washington State during necropsies of beached/dead whales.

5) Discuss whether your project involves activities known or suspected of introducing or spreading invasive species, intentionally or not, (e.g., transporting animals or tissues, discharging ballast water, use of equipment at multiple sites). Describe measures you would take to prevent the possible introduction or spread of non-indigenous or invasive species, including plants, animals, microbes, or other biological agents.

Not applicable; we will be working in a museum and/or laboratory setting. Museum plugs are typically preserved in formalin or dry and sealed in a glass container and therefore do not pose a threat to spreading invasive species.

Project Contacts

Primary Contact: Stephen John Trumble

Principal Investigator: Stephen John Trumble

Other Personnel

Name	Role(s)
Sascha Usenko	Co-Investigator

Attachments

Contact - Sascha Usenko (Added Nov 18, 2021)
Contact - Stephen John Trumble (Added Sep 9, 2021)
Project Description - (Added Oct 8, 2021)
References - (Added Oct 8, 2021)

Status

Application Status: Application Complete
Date Submitted: September 9, 2021
Date Completed: October 7, 2021
FR Notice of Receipt Published: October 15, 2021 **Number:** 2021-22539
Comment Period Closed: November 15, 2021 **Comments Received:** No **Comments Addressed:** No
Last Date Archived: November 23, 2021

- **MMPA/ESA Parts permit**

Current Status: Issued **Status Date:** November 23, 2021
Section 7 Consultation: No Effect
NEPA Analysis: Categorical Exclusion
Expire Date: November 30, 2026

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Modification Requests

Reports